HEPATITIS B VACCINATION USING A DISSOLVABLE MICRONEEDLE PATCH

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HEPATITIS B VACCINATION USING A DISSOLVABLE MICRONEEDLE PATCH

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If I have seen further it is by standing on the shoulders of giants. – Isaac Newton

To my parents, for encouraging me to venture out into the world.

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LIST OF SYMBOLS AND ABBREVIATIONS

ANOVA	Analysis of Variance
Anti-HBs	Hepatitis B Surface Antibodies
CMC	Carboxymethylcellulose
ELISA	Enzyme-Linked Immunosorbent Assay
GAVI	Global Alliance for Vaccines and Immunization
HBsAg	Hepatitis B Antigen
HBV	Hepatitis B Virus
IM	Intramuscular
MN	Microneedle
MNP	Microneedle Patch
P	Probability to determine statistical significance
WHO	World Health Organization

SUMMARY

Despite improved vaccination rates against hepatitis B, there remain critical barriers to addressing gaps in vaccination coverage. The need of an effective supply chain, vaccine waste management, trained healthcare providers and cost are all issues that impede mass vaccination campaigns around the world. Microneedle patches have been proposed as an alternative mode of vaccination. Microneedle patches consist of micron-scale projections that are capable of disrupting the stratum corneum by creating holes in the skin to deliver therapeutic agents. Small and lightweight, microneedle patches are a promising alternative to the bulky multi-dose vials and syringes currently used in mass vaccination campaigns. Furthermore, the high density of antigen presenting cells in the the skin make transcutaneous immunization via microneedles advantageous, as they target vaccine cargo to the topmost layer of the skin.

The key goal of this project was to develop a microneedle patch for hepatitis B vaccination that is simple to administer and of comparable immunogenicity to conventional intramuscular vaccination. Trehalose was used as a stabilizing excipient for both coated metal and dissolvable microneedles. Moreover, patches were used in vivo to compare the elicited immune response in both mice and rhesus macaques. Additionally, the mechanical properties of our microneedle patch were evaluated via both theoretical and experimental approaches to predict failure force. This work shows that microneedle patches can successfully encapsulate and deliver hepatitis B antigen to generate a strong and sustained immune response in multiple animal models.

CHAPTER 1. INTRODUCTION

1.1 Motivation

Hepatitis B is a viral infection that affects the liver, with the potential to progress to chronic disease and cirrhosis. Despite vaccines being available for over three decades, global prevalence of chronic carriers is still as high as 20 %.[1] Currently, hepatitis B vaccination first occurs at birth (as recommended by the CDC), and coverage for infants worldwide is at 83%[2] - up from 75% in 2010.[3] In general, the main hindrances to vaccination campaigns are cold chain requirements, training of health professionals and complicated logistics stemming from bulky shipments of multi-dose glass vials and syringes.[4] Limited financial resources or personal preference may also be a driver of home births worldwide, resulting in a missed opportunity for infant vaccination.[5] While hepatitis B vaccine retains significant activity at controlled exposures to heat[6], the possibility of a dry, temperature stable, and easy to administer vaccine could be the key to improving vaccination efforts. Furthermore, because many mothers worldwide don't give birth in a health facility, an easily portable delivery method is essential to improve compliance with dosing regimens that require the first dose to occur at birth and typically administered by minimally trained birth attendants.[4,7]

1.1.1 Hepatitis B (disease)

Hepatitis B Virus (HBV) is a viral infection that targets the liver. A major global health issue, HBV is most prevalent in Southeast Asia, the Middle East and sub-Saharan

Africa[63] (Figure 1). Approximately 4.5 million new HBV infections occur worldwide each year.[8]

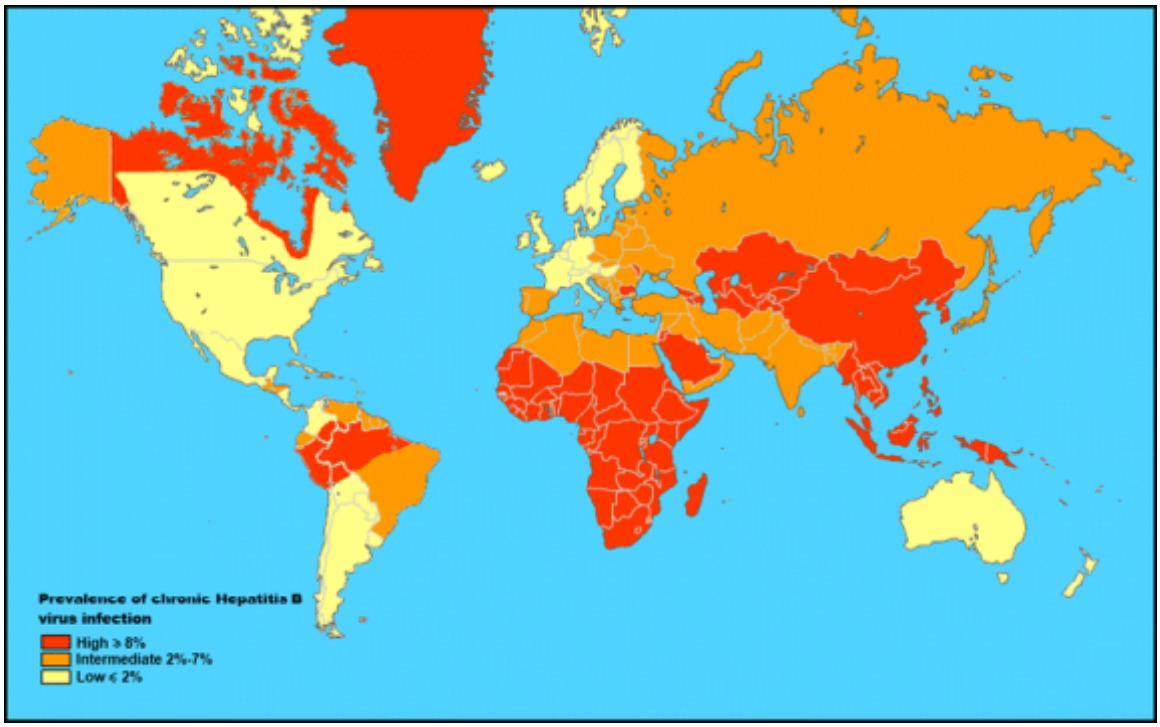

Figure 1:Prevalence of chronic hepatitis B infection. Areas with > 8% are shown in red and considered to be areas of high HBV prevalence. Reproduced from Lebre, et.al.[9]

In the US alone, approximately 0.7-1.4 million people have chronic hepatitis B infection.[10] With 40% of people globally in contact with or identified as carriers [9], HBV can spread by percutaneous or mucosal exposure to infectious blood or body fluids such as saliva, vaginal or seminal fluids. [3]

HBV can cause both acute and chronic (> 6 months) infections – symptoms include fever, fatigue, jaundice, abdominal pain and vomiting. Between one-third and one-quarter of persons infected chronically with HBV are expected to develop long-term consequences, such as cirrhosis and liver cancer.[9]

1.1.2 Coverage of Vaccination

The vaccination schedule for hepatitis B consists of a three-dose series. In the US, reported hepatitis B vaccination coverage (≥3 doses) varies widely by age group – 32.2% among adults aged 19-49 years, and 15.7% among adults aged ≥50 years.[11] Globally, HBV vaccination coverage is much lower.[65]

1.2 Objectives

The objectives of this study are to fabricate a dissolvable microneedle patch for hepatitis B vaccination. The mechanical properties of these patches were evaluated using theoretical and experimental approaches. Immunogenicity of these patches was evaluated initially in female, BALB/c mice, which was followed by a study in rhesus macaques.

CHAPTER 2. BACKGROUND

2.1 Hepatitis B Vaccine

Hepatitis B vaccine consists of a liquid suspension of purified hepatitis B surface antigen (HBsAg), adsorbed onto some type of aluminum salt. The first vaccines developed were plasma-derived and made commercially available in 1982.[12] Currently, both plasma-derived and recombinant protein vaccines are on the market, with the latter being most prevalent. Most licensed recombinant hepatitis B vaccines are a 226 amino acid product of the S gene of HBV [13,62] – the determinant primarily responsible for immune response is exposed on the particle surface, same as is the case for the natural HBsAg particle. The only significant difference is the naturally occurring glycosylation in the natural HBsAg particle.[3]

2.1.1 Vaccine Structure

The HBV virus contains surface proteins (denominated small [S], medium [M] and Large [L]), which are produced in excess amounts during infection and also circulate in the blood as 22-nm spherical and tubular particles.[14] In recombinant vaccines, usually the sole protein present is the S protein, which accounts for 60% of natural HBsAg particle mass.[15] S proteins appear as spike-like protrusions at the particle surface. The "a" determinant on top of the S protein has been demonstrated to contain at least three different antigenic sites, and is therefore considered to be the dominant with regards to immunogenicity.[15,16]

2.1.2 Vaccine Stability

The recommended storage temperature for hepatitis B vaccine is between 2º and 8º C.[64] The vaccine is generally stable for 3 to 4 years from the date of manufacture if stored in this temperature range.[17] However, the stability of hepatitis B vaccines from different manufacturers can vary considerably.[18] Most hepatitis B vaccines are relatively heat stable and have only a small loss of potency when exposed to heat. Previous work has shown that current commercial formulations can still elicit protective titers after 1 week at 45^{o}C, or up to several weeks at 40^{o}C.[18]

Given the logistical issues that frequently plague immunization efforts world-wide, many have sought to explore transport-friendly alternatives to liquid formulations. Spray-freeze drying is one approach used by some to eliminate cold chain dependence. Tonnis, et. al. showed that spray-freeze dried HBsAg in combination with sugars like inulin or dextran and trehalose resulted in improved stability and higher IgG immune responses for intramuscular administration in comparison to liquid HBsAg.[19] The powder formulations in this study retained 90% of their immunogenicity after 3 months at 60^{o}C. The use of sugars has been long known to lend a stabilizing effect when used as an excipient within a vaccine solution, for both liquid and solid formulations.[7,20]

2.2 Microneedle Patches

2.2.1 Microneedle Patch Technology

One challenge for intradermal delivery is the need to increase the amount of biologicals that can be delivered via the skin. Microneedle patches consist of an array of tapered projections which can be applied to the skin to pierce the stratum corneum, and therefore address this challenge by coupling physical disruption with the simultaneous delivery of a drug cargo into the skin. Microneedles can be classified per the following categories: solid, coated, hollow and dissolving (Figure 2:). Solid, coated and hollow microneedles are typically made out of materials like silicon, ceramic and metal[21] whereas dissolvable microneedles rely on a water-soluble matrix that typically encapsulates the drug cargo of choice. The training required for application of a microneedle patch is minimal in comparison to other methods, and application is generally considered to be painless.[22]

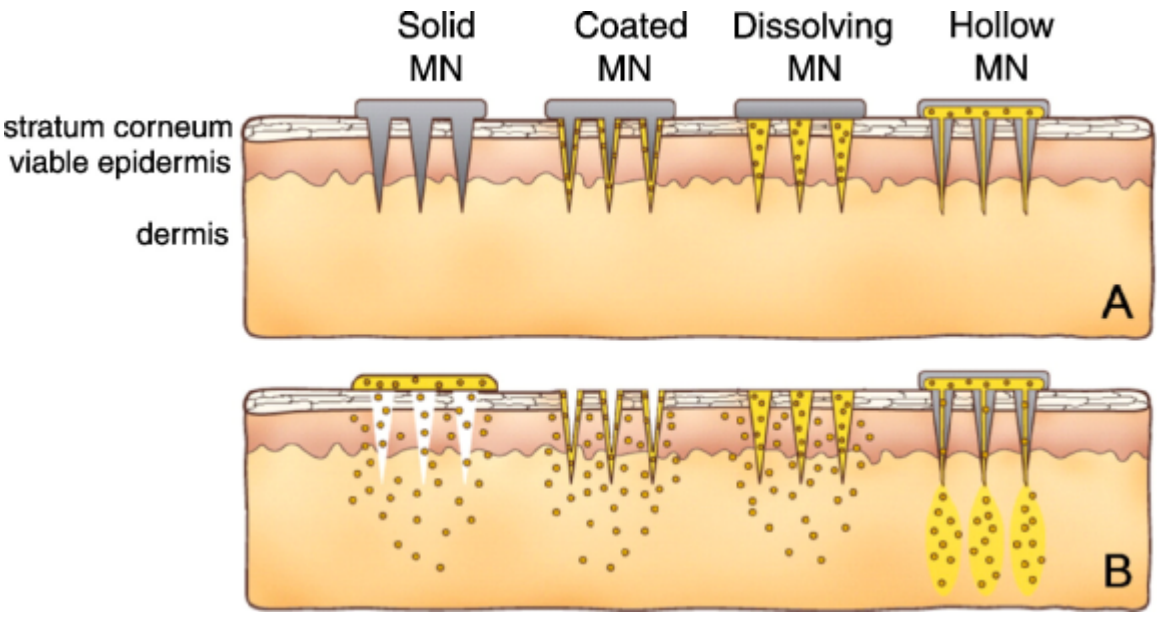

Figure 2: Types of microneedle patches. Adapted from Kim, et.al [23]

Solid microneedles create holes in the skin after which a drug will diffuse from a reservoir into the channels created within the epidermis. This "poke and patch" approach

has been used for varied applications[24], but is often limited by the same supply chain requirements that hinder traditional immunization procedures. Similar to hypodermic injection, hollow microneedles rely on a lumen through which a drug solution will flow into the skin. Drug reservoirs can be pressurized to modulate flow rate and subsequently the delivery profile of the drug of interest.[23] Both solid and hollow microneedles can provide an easier way to transition existing drug formulations into the microneedle delivery space, though as mentioned earlier, they pose many of the drug stability and logistical issues associated with traditional IM immunization.

Coated microneedles combine physical disruption of the skin while carrying the drug cargo in the form of a coating on its surface. This involves creating a formulation suitable for coating that can maintain therapeutic activity throughout the coating process and subsequent dissolution into skin. While limited in the amount of drug that can be applied onto the microneedle array, this approach presents the advantage of easing supply chain constraints, by bypassing the need for cold storage temperatures and the handling of liquid vaccines.

Microneedles can also be made in such a way that they encapsulate heat-sensitive proteins while being rigid enough to pierce skin. This is the case for dissolvable microneedles, which incorporate the drug of interest within their microneedle matrix, as well as other water-soluble materials that provide sufficient mechanical strength to pierce skin and dissolve within minutes to deliver a drug cargo. The use of sugar-based excipients can protect heat-sensitive proteins during the casting process, and because microneedles can be fabricated at room temperature with no harsh solvents, drug activity can be maintained.[25] Furthermore, dissolvable microneedles leave no biohazardous

sharps waste, can be manufactured at low cost, and their thermostability poses an additional advantage for vaccination campaigns.

2.2.2 Vaccination with Microneedle Patches

The epidermis and dermis possess immunocompetent cells such as Langerhans and dermal dendritic cells which assist in adaptive immunity (Figure 3). By incorporating a vaccine within the constitutive microneedle matrix or onto the microneedle surface, the vaccine can be brought into contact with these antigen presenting cells (APCs)[26] to elicit an immune response. Since a microneedle's dimensions are small, there is less risk of

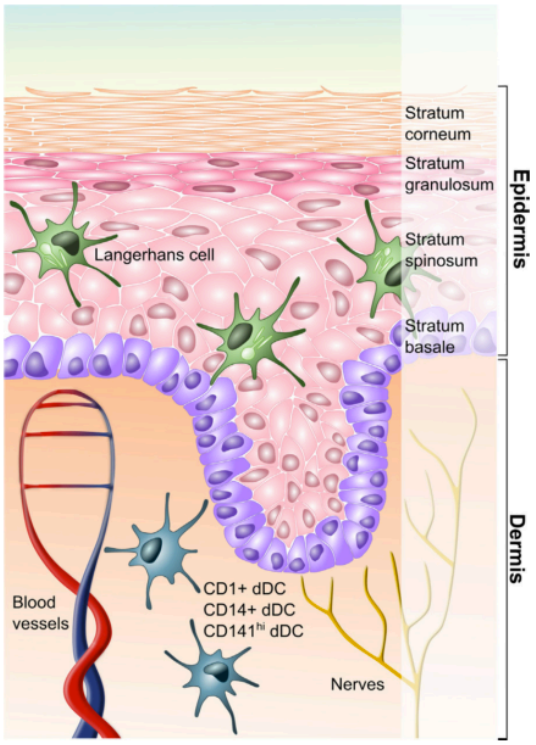

Figure 3: Antigen presenting cells within layers of skin. Stratum corneum is 10-20 µm thick. Epidermis and dermis thickness are around 100 µm and 0.3-4 mm, respectively. Adapted from Engelke, et. al. [26]

error for targeting vaccination in the skin in comparison to the Mantoux technique, where a 26 or 27 gauge needle is inserted at an angle into the skin.[27] This technique is particularly challenging to administer consistently. Furthermore, the high density of APCs within the skin lends microneedles the potential of generating a strong immune response at lower doses than traditional vaccination regimens.[28]

In recent years, numerous studies have been published on the use of microneedle patches as a vaccination strategy. Typical administration can take between a few minutes to a few hours, depending on the type of microneedle and formulation.[29–31] In vivo studies have demonstrated the efficacy of hollow microneedles for dermal administration of inactivated poliovirus vaccine[32] in rats. Other examples of intradermal vaccination via hollow microneedles is the FDA-approved flu vaccine developed by Sanofi[33], as well as temperature-stable, dissolvable microneedle patches have been developed for live, inactivated, subunit and DNA vaccines such as those for measles[34] , influenza[23] and rabies.[35,36] Notably, dissolving microneedle patches containing influenza vaccine with calcium heptagluconate and arginine as excipients were shown to retain vaccine stability for up to 24 months at room temperature.[37]

2.2.3 Potential Impact of Microneedle Patch Vaccination on Public Health

In 2005, the CDC submitted recommendations to increase vaccination coverage by implementing a birth dose of hepatitis B vaccine in medically stable infants.[38] Because many births worldwide occur away from health facilities and medically trained employees, this represents a significant gap in coverage not easily addressed by current methods. In 2011, it was found that 37.3-89.9% of the poorest women in developing

countries give birth in a home setting.[5] Furthermore, in cases of births given by chronic HBV carriers – estimated to be around 25,000 per year in the US[39]- perinatal HBV transmission can be prevented if vaccination occurs within the first 12 hours of an infant's life. Thanks in part to its size and simplicity, a microneedle patch could address this need for immediacy and accessibility. Other obstacles for improving vaccination coverage include: the need for effective supply chain, vaccine wastage from multi-dose vials, need for healthcare provider training, cost and the risk of needle re-use.[4] The introduction of microneedle patches as an alternative mode of immunization would potentially bypass most if not all of these issues.

Skin vaccination has been shown to produce similar or higher immune responses compared to intramuscular injection, with dose-sparing effects presented in some cases.[40–42] Given that 10% of the general population shows poor immunological response to HBV vaccination [43,44], transdermal delivery poses as a potential advantage to patients with impaired immune response. Dose sparing has also been observed in some microneedle patch applications, which could help maximize coverage under strained manufacturing.

In many cases, the cost of transport and other logistics can double or triple the cost of vaccine administration.[4] Many academic and commercial ventures are interested in transdermal vaccine dosage forms that allow for transport and storage at room temperature in a dry state. The incorporation of excipients like sugars allow for hydrogen bond formation that is believed to stabilize vaccines by replacing the protein-solvent interactions in its liquid state.[7]

The simplicity and minimal risks of microneedle patch application means that the dose is already contained and no additional intervention with the antigenic material is required. The ability to reconstitute and administer at the same time can minimize reconstitution errors, as well as the training needed to safely administer vaccine. Dissolvable microneedles eliminate concern over sharps collection and needle-stick injuries, eliminating unsafe injection practices that put both health workers and patients at risk. Finally, in the case of countries were patient compliance is an issue, it has been shown that offering a microneedle patch as an alternative could increase intent to vaccinate by 17%.[22]

2.3 Transcutaneous Immunization Against Hepatitis B

Though different from microneedles, liquid HBsAg has already been successfully administered intradermally via the use of disposable syringe jet injectors (DSJI) for dermal vaccination in the minipig model.[45] Several initial studies for transcutaneous immunization via microneedles against HBV have focused on leveraging the "poke and coat" approach. In this case, metal microneedles are used as a pretreatment, followed by the topical application of a vaccine-containing solution. One study using this approach developed elastic HBsAg-associated vesicles which were applied following microneedle pretreatment, and successfully elicited antibody responses in mice.[46] Using a similar approach, Guo et. al incorporated HBsAg into carbomer-based hydrogels which were applied to mice after pretreatment with solid silicon microneedles.[47] Similarly, scraping the skin with blunt microneedles has also been used as a technique to deliver DNA vaccine against HBV.[48]

More recently there has been interest in developing solid vaccine formulations for HBsAg. Coated metal microneedles have been used to deliver bulk HBsAg intradermally by incorporating polydi(carboxylatophenoxy)phosphazene as both an excipient and immuno-adjuvant.[49] In 2015, cationic liposomes encapsulating HBV DNA vaccine were delivered via dissolving microneedles in mice.[50] Until now, there has not been any work incorporating the recombinant HbsAg particles within a completely dissolvable microneedle matrix.

2.4 Mechanical Testing of Microneedles

In order to ensure efficient drug delivery, microneedles must be able to deliver vaccine into the target tissue without mechanical failure. Reliable and reproducible insertion depends on microneedles being able to sustain the loads involved during administration. In order to understand the loading limits of microneedles, previous studies have evaluated the mechanical behavior of microneedles in relation to beam failure theory.[51] Microneedles are thus treated as columns which can fail in two different ways: crushing and buckling. A column with a cross sectional dimension in the vicinity of its length (short column), is more likely to be crushed, whereas columns with higher aspect ratios fail by buckling.

2.4.1 Buckling Failure

Buckling occurs when elastic or inelastic stability of the structure causes the structure to fail sideways. Modeling buckling has been treated as an eigenvalue problem, where the solution calculation yields a multiplying factor which can be used to scale the force applied to that which would be necessary for buckling failure to occur. Many

analytical solutions exist in the literature [52,53] for different common structural configurations.

In the case of elastic instability, the Euler equation provides a prediction of the critical load required for buckling:

$$\boldsymbol{P_{cr}} = \frac{\boldsymbol{\pi^2 EI}}{\boldsymbol{L_e^2}} \quad (1)$$

L_e refers to the effective length (L_e=KL) where K is a factor that depends on how the microneedle ends are restrained and L is the actual length of the column.[51] The K values are 1.0, 0.7 and 0.5 for pinned-pinned, pinned-fixed and fixed-fixed columns respectively. E represents the tensile or Young's modulus and I is the moment of inertia as calculated below:

$$I = \frac{\pi D^4}{64} \quad (2)$$

Where D is the equivalent diameter as calculated by Park et. Al[48]:

$$D_{eq} = D_{tip} + \frac{D_{base} - D_{tip}}{3} \quad (3)$$

2.4.2 Insertion Forces

The forces a microneedle is subjected to during insertion into skin will vary according to substrate, application method and microneedle geometry. Currently, most microneedle patches used for research are applied manually, but most patches under commercial development use of force-driven applicators. These may apply an axial force to the microneedle patch that is significantly higher than the minimum insertion force in

order to maximize insertion and minimize errors. Representative forces for current applicators vary from 3–22 N for patches containing hundreds of sharp-tipped microneedles [54], whereas insertion of blunt-tipped, hollow microneedles required 0.1-3 N per microneedle.[55] Consequently, microneedles must be tough enough to withstand a variety of forces.

CHAPTER 3. METHODS

3.1 HBsAg Quantification and Antibody Titer Measurements

HBsAg quantification and antibody titers of rhesus macaque serum samples were measured using the Vitros Immunodiagnostic System (Ortho Clinical Diagnostics, Raritan, NJ) and were carried out at the Centers for Disease Control and Prevention (CDC). An immunometric immunoassay technique is used, where HBsAg in the sample reacts with mouse monoclonal anti-HBs coated onto wells and with a horseradish peroxidase-labelled mouse monoclonal anti-HBs in the conjugate. Bound conjugate is measured by a luminescent reaction. Mice serum samples were analysed with a commercial anti-HBs ELISA kit (Bio-Rad, Hercules, CA).

3.2 Concentration of Hepatitis B Vaccine

Concentrated bulk solution of HBsAg was generously provided by the Serum Institute of India (Pune, India). Starting antigen concentration was 2.38 mg/mL. This bulk vaccine was concentrated using Vivaspin 20 centrifuge spin filters with a 10 kDa molecular weight cutoff (GE Life Sciences, Pittsburgh, PA) by centrifugation at 700 g for 2 min. Vaccine bulk was concentrated 4-fold and 5.4-fold for the dissolvable and the metal microneedles, respectively. HBsAg concentration was measured via the Vitros Immunodiagnostic System.

3.3 Microneedle Fabrication

3.3.1 Coated Microneedle Patches

In plane rows of laser-cut stainless-steel microneedle arrays were coated using an in-house dip-coating device. The microneedle arrays were fabricated by Tech Etch (Plymouth, MA). Each array consisted of 5 microneedles measuring 750 µm in length and 170 µm by 50 µm in base cross-sectional area, and arranged in a linear array with 1.6 mm microneedle tip-to-tip spacing. Two linear micro-positioners allowed for alignment and dipping of individual microneedles into a micro-pipette tip containing a coating solution. Coating solution consisted of concentrated vaccine mixed 1:1 with a solution of 2% w/v carboxymethylcellulose (CMC, Sigma–Aldrich, St. Louis, MO) and 20% w/v trehalose (Sigma–Aldrich). This device design allowed for coating of only the microneedle shaft, thereby avoiding contamination of the array base. Immunoassay analysis showed that 1.3 ± 0.1 µg of HbsAg was coated onto coated microneedle patches for our mice study.

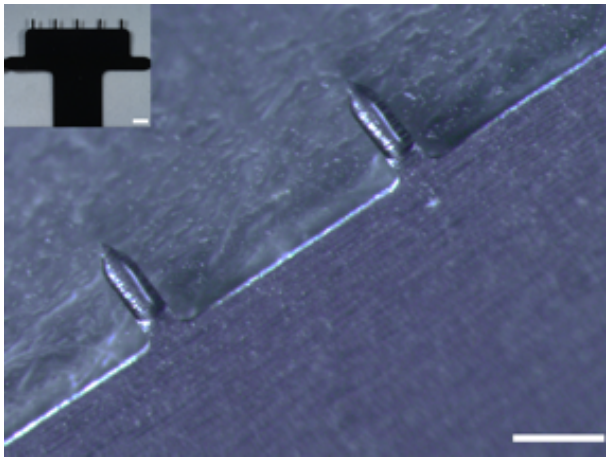

Figure 4: Close-up of coated microneedle patch. Scale bar is 500 µm. Inset: Coated microneedle patch containing 5 microneedles. (Scale bar: 1mm)

3.3.2 Dissolvable Microneedle Patches

Microneedle patches were fabricated as described previously[34,56]. In this study, the concentrated hepatitis B vaccine stock was mixed into a casting solution at a 60:40 ratio with a solution containing 25% w/v trehalose and 2.5% w/v CMC. For our initial mice study, unmodified bulk HBsAg (2.38 mg/mL) was used instead of the concentrated HBsAg solution. Molds used to fabricate microneedle patches consisted of a 10 x 10 array of conical cavities in the shape of microneedles, and 35 μL of the vaccine/casting solution was applied to each mold. After an hour at room temperature (20 – 25 °C) and humidity (20 – 50% rh), a second casting was applied to form a patch backing. This solution consisted of sucrose (Sigma–Aldrich) and poly-vinyl alcohol (Acros Organics, New Jersey USA) dissolved in deionized water to a final concentration of 0.53 mg/mL of each solute at 25°C for 1 h. Vacuum was applied to the mold at room temperature for 3 h. After that, molds were allowed to further dry in a desiccator at room temperature for 2 days. Patches prepared for mechanical testing experiments did not contain HBsAg, and phosphate-buffered saline (PBS) was used in lieu of vaccine solution.

Patches were removed from the mold by applying a polymethylmethacrylate (McMaster-Carr, Atlanta, GA) disk covered with double-sided tape (MacTac, Stow, OH) to the back of the mold and slowly pulling the patch away from the mold. Upon removal from their molds, patches were stored inside a sealed pouch with desiccant (Drierite, Xenia, OH) at 4°C for less than 24 h until use. To avoid damaging the microneedles through condensation of atmospheric water, pouches were allowed to return to room temperature prior to patch removal and application.

Immunoassay analysis showed that 0.9 ± 0.3 µg and 12.8 ± 4.0 µg of HBsAg was encapsulated in each dissolvable microneedle patch for our mice and macaque study, respectively.

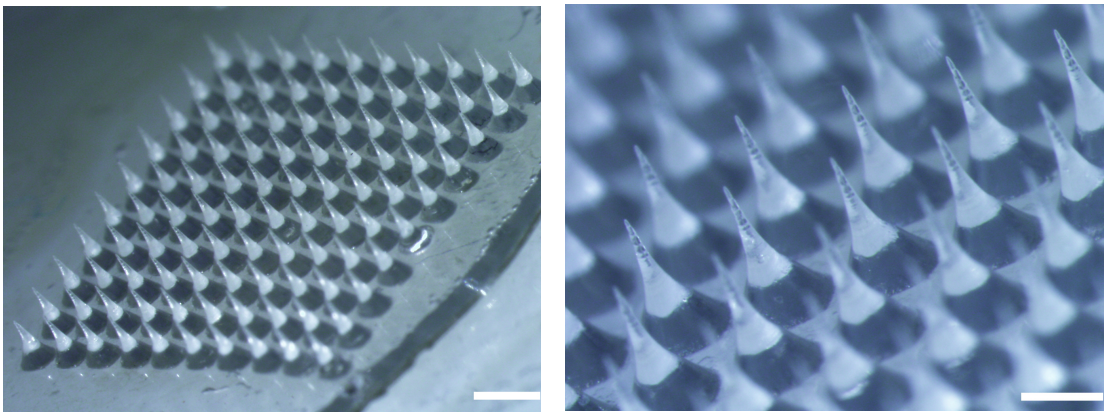

Figure 5: Dissolving HBsAg microneedle patches. Left: Picture of a 10 x 10 dissolvable microneedle patch. Scale bar is 1mm. On right: Magnified view of conical microneedles. Scale bar on right is 500µm.

3.4 Mechanical Characterization

3.4.1 Force-Displacement

Microneedle patches were kept inside sealed pouches with desiccant until testing. Failure forces of 10 x 10 microneedle patches under an axial load were measured utilizing a digital force gauge (MARK-10 Series 5, Copiague, NY). Force-displacement curves were generated by measuring both force and displacement as the test station's moving crosshead pressed against the microneedle array at a rate of 1.0 mm/min. Upon reaching a specific load, the crosshead was immediately lifted. Microneedle patches were

then stored inside their original pouches with desiccant – though not sealed – for at least 24 hours before subsequent insertion into skin.

3.4.2 Microneedle Insertion

Sections of frozen porcine skin stored in resealable plastic bags were thawed by placing in water (while still inside bag) and then placed on absorbent pads to dry under air. A Kimwipe (ThermoFisher, Waltham, MA) was placed on top of the skin section to determine if the skin surface was dry. Skin was used within 20 minutes of removal from the plastic bag, and only if the Kimwipe indicated a dry skin surface. Microneedle patches previously subjected to a specific force load were applied to the skin held under tension while applying a force of approximately 7 N and held for 30 seconds. After 20 minutes, the MN patch was removed and gentian violet dye (HUMCO, Texarkana, TX) was applied to the skin to stain puncture spots. The skin was imaged using an Olympus SZX16 microscope (Tokyo, Japan) to determine insertion efficiency.

3.5 Mice Study

The immunogenicity of hepatitis B vaccine patches was tested in female, 11 week old BALB/c mice. They were divided into five groups of 8 animals each. Groups were labeled in the following manner: (1) Intramuscular Bulk Vaccine, (2) Reconstituted Dissolvable Microneedle Patch, (3) Reconstituted Coated Microneedle Patch, (4) Dissolvable Microneedle Patch (dMNP) and (5) Coated Microneedle Patch (cMNP). The mice were allowed at least 2 weeks to acclimate before beginning the study. Animals were anesthetized using isoflurane during vaccination and blood collection. For animals receiving MN patch administration, their backs were shaved using electric shears

followed by application of a depilatory cream (Nair, Princeton, NJ) 1 day before vaccination. All animals received two vaccine doses (by the same method of delivery) separated by three weeks.

For intramuscular injection in group 1, stock vaccine was diluted using sterile PBS to administer a dose of 2.5 μg HBsAg contained within 25 μL. This solution was injected intramuscularly using a 29 gauge hypodermic needle on the animal's hind leg. For group 2, 10 dMNPs (each containing 0.9 ± 0.3 μg HBsAg) were dissolved into 270 μL of PBS, of which 25 μL containing a dose of 0.8 ± 0.1 μg HBsAg was injected. For group 3, 20 cMNPs (each containing 1.3 ± 0.1 μg HBsAg) were reconstituted in 300 μL of PBS, of which 25 μL containing a dose of 2.2 ± 0.04 μg HBsAg was injected. For group 4, two dMNPs were applied sequentially to the hairless skin section and left on the animal for 10 min per patch to deliver 0.9 ± 0.2 μg HBsAg (based on an expected delivery efficiency of 50%).[57] For group 5, three cMNPs were applied sequentially to the hairless skin section and left on the animal for 10 min per patch to deliver 1.3 ± 0.1 μg HBsAg (based on an expected delivery efficiency of 50%). The variability in HBsAg dosing levels between groups was due to initial difficulties with analytical methods.

Visual evidence of skin punctures by MNPs was noticeable (to a trained eye) immediately after administration; no bleeding was observed. On some animals, small scabs developed from depilatory cream residue left on the skin in initial studies. Care was taken to avoid leaving residue during subsequent hair removal. Subsequent patch application occurred on intact skin, avoiding the scab area. Mice were re-shaved and depilated prior to the second administration, because fur grew back to normal during the 3 weeks between first and second administration. Blood was collected on weeks 2, 4, 5

and 8 to measure antibody titers (i.e., anti-hepatitis B surface antigen (HBs) titer as determined by Spradling, et.al performed at CDC Reference Laboratory).[58,59] After week 8, the animals were sacrificed by isoflurane euthanasia. The protocol for these experiments was approved by the Institutional Animal Care and Use Committee (IACUC) at Georgia Tech.

3.6 Macaque Study

The immunogenicity of the hepatitis B vaccine MNPs was tested in rhesus macaques. Animals were divided into three groups of 4 monkeys each. Group I was vaccinated intramuscularly with 10 μg unformulated stock vaccine (no adjuvant) and Group II was similarly injected with 10 μg of a commercial hepatitis B vaccine formulation containing alum adjuvant (Serum Institute of India). Group III received hepatitis B vaccination via application of three dMNPs (each containing 12.8 ± 4.0 μg HBsAg) that administered 24 ± 8 μg (based on a 63% delivery efficiency; see below), respectively.

Animals were anesthetized via ketamine injection during vaccination and collection of blood. For animals receiving microneedle patch administration, their backs were shaved using electric shears followed by application of a depilatory cream (Nair). Patches were applied to the hairless skin section and left for 20 minutes. Additional hair removal using shears and depilatory cream was done prior to boost. No evidence of scabbing due to depilatory cream application was seen.

Visual evidence of skin punctures by microneedle arrays was noticeable (to a trained eye) immediately after administration; in a few cases, trace blood residue was

observed on the sites of a few MNs on used patches. Blood was collected weekly and analyzed for antibodies, The protocol for these experiments was approved by the IACUCs at CDC and Georgia Tech.

To confirm the dose delivered to the skin of the macaques, residual HBsAg in the patches used for the second dose was measured post-administration and subtracted from the original antigen loading. Based on these data, delivery efficiency (the ratio of delivered antigen to loaded antigen) was determined to be 63 ± 16 %.

3.7 Statistics

Statistics were performed using Prism software version 7 (GraphPad, La Jolla, CA). P values < 0.05 were considered significant. A two-way ANOVA with a Tukey post-test was used to compare multiple samples.

CHAPTER 4. RESULTS

4.1 Mechanical Characterization

Stainless steel MNs have been shown to have sufficient mechanical strength to penetrate into skin[40,60–62] and therefore have not been mechanically characterized here. However, dissolvable MNs are typically not as strong and their design is strongly influenced by mechanical strength considerations. Therefore, the mechanical behavior of dissolvable microneedle patches were characterized to relate the failure of these microneedles as determined by force-displacement curves analyzed in the context of beam theory.

4.1.1 Measurement of Microneedle Failure

Mechanical behavior of microneedle patches was studied by generating force-displacement curves obtained by pressing patches containing a 10 by 10 array of dissolvable microneedles against a rigid surface. In general, force increased with increasing applied displacement, but the response could be divided into two regions (Figure 6). Based on 11 replicate measurements, we found that at small displacement (i.e., less than 0.15 ± 0.05 mm) there was a roughly linear increase in force that led to an apparent yield point at 1.1 ± 0.6 N of axial force applied to 100 microneedles (i.e., 11 ± 6 mN per microneedle).

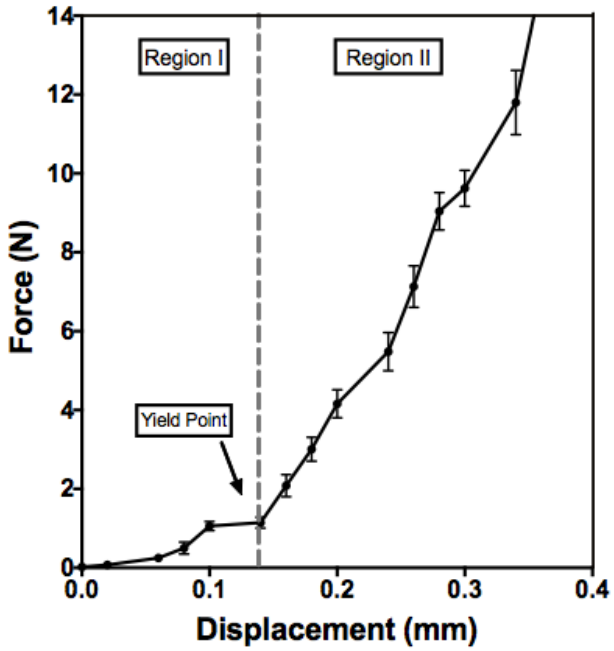

Figure 6: Representative force-displacement curve for a 10x10 microneedle patch. Data shown is for a single dMNP.

Microneeedles were imaged following application of different axial forces. Unused microneedles showed a continuously tapered conical structure and a sharp tip (Figure 7a). After application of 5 N (above the transition from the first to second region), initial evidence of microneedle tip deformation could be seen (Figure 7b). However, at higher forces of 15, 25, 50 and 75 N (Figures 7c,g,h,i), progressively greater microneedle tip deformation was seen. Under the conditions used, microneedle fracture was not seen.

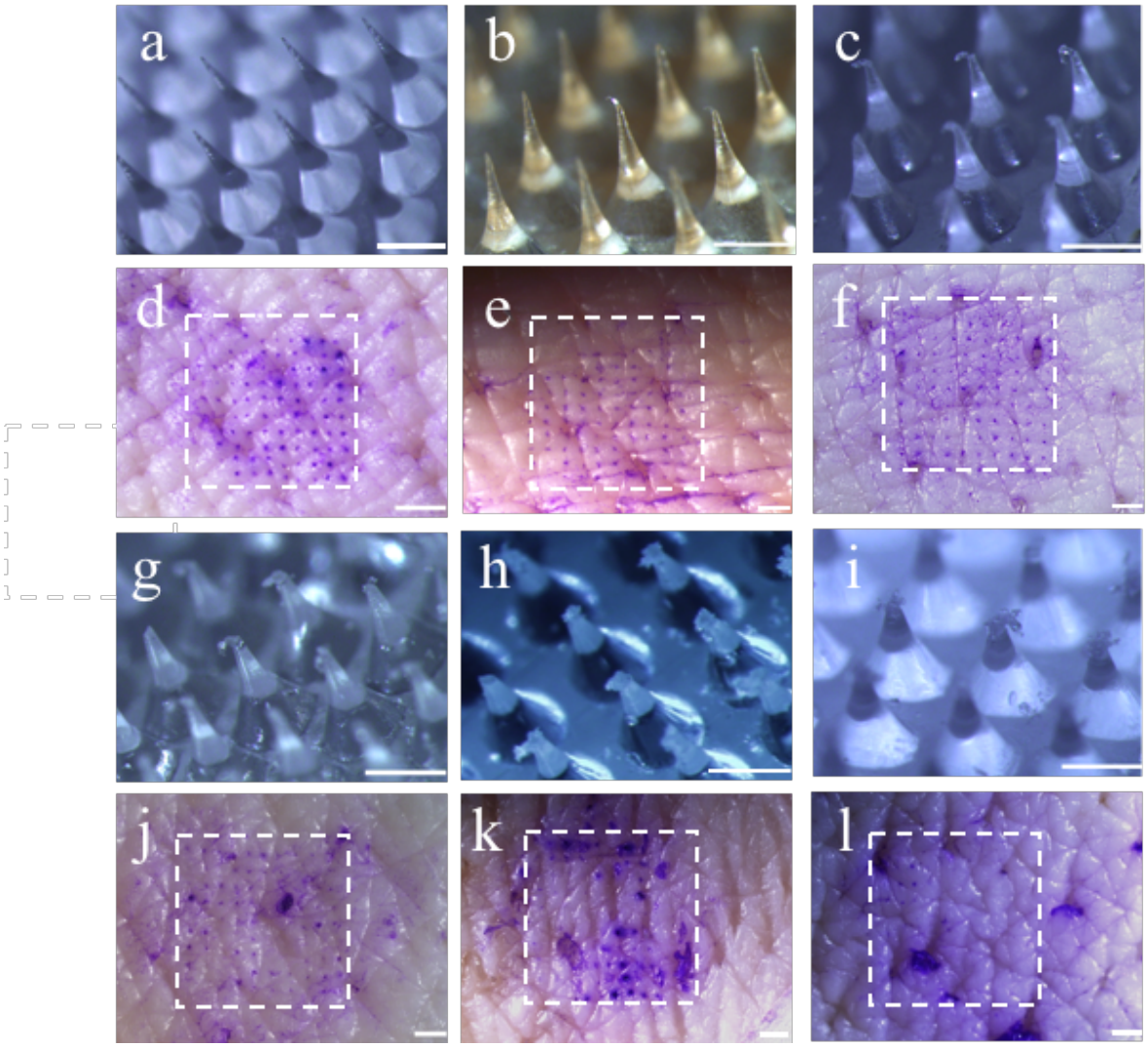

Figure 7: Microneedle deformation (from left to right: 0, 5, 15, 25, 50 and 75 N of applied force) and subsequent insertion into ex-vivo pig skin site of patch application shown in box. Scale Bar (a-c, g-i): 500 µm. Scale bar (d-f, j-l): 1mm

Comparing the forces associated with changes in measured mechanical properties (Figure 6) and with morphological changes observed microscopically (Figure 7), it appears that the mechanics in Region I of the force-displacement curve correspond to the behavior of intact microneedles and the yield point which corresponds to the point of initial deformation of the microneedles (occurring between Figures 7a and 7b). Region II

appears to correspond to progressively increasing microneedle tip deformation (occurring between Figures 7b and 7c) and, at higher forces microneedle tips appear crushed and the matrix material begins to fan out in a radial direction (Figures 7g-i),.

While microneedle tip deformation is not good for insertion into skin, it may be that some deformation is acceptable. We therefore assessed the ability of microneedles to penetrate into skin after different levels of tip deformation. Intact microneedles were able to penetrate the skin efficiently, as indicated by an assay that stains sites of microneedle penetration into skin (Figures 7d-f, j-l). At 5 N, there was minimal damage to the tips and insertion efficiency remained high (Figure 7e). At 15 N, minor chipping and hooking of the tip occurred. However, subsequent insertion of the microneedle array into skin was only slightly reduced compared to that of an intact patch (Figure 7f). At 25 N and 50 N, there was increased bending and tip deformation that correlated with a loss of insertion efficiency, as evidenced by the reduction of penetration holes (Figure 7j-k). Finally, at 75 N, microneedle tips were crushed and no longer able to penetrate skin (Figure 7l). These data are quantified in Figure 8a.

The reduction of microneedle height was measured by comparing microscopic images of stressed microneedles to intact microneedles (e.g., Figure 7a-c,g-i). With increasing force, there were greater reductions of microneedle height initially but then microneedle height plateaued at a value of approximately 50% height loss (residual height approximately 300 μm) (Figure 8b). The increase in microneedle tip diameter that accompanies loss of height results in a blunt tip which could hinder insertion, though crushed tips with up to 200 μm height loss (i.e., patches compressed with up to 15 N) still had relatively reliable insertion (Figure 8a).

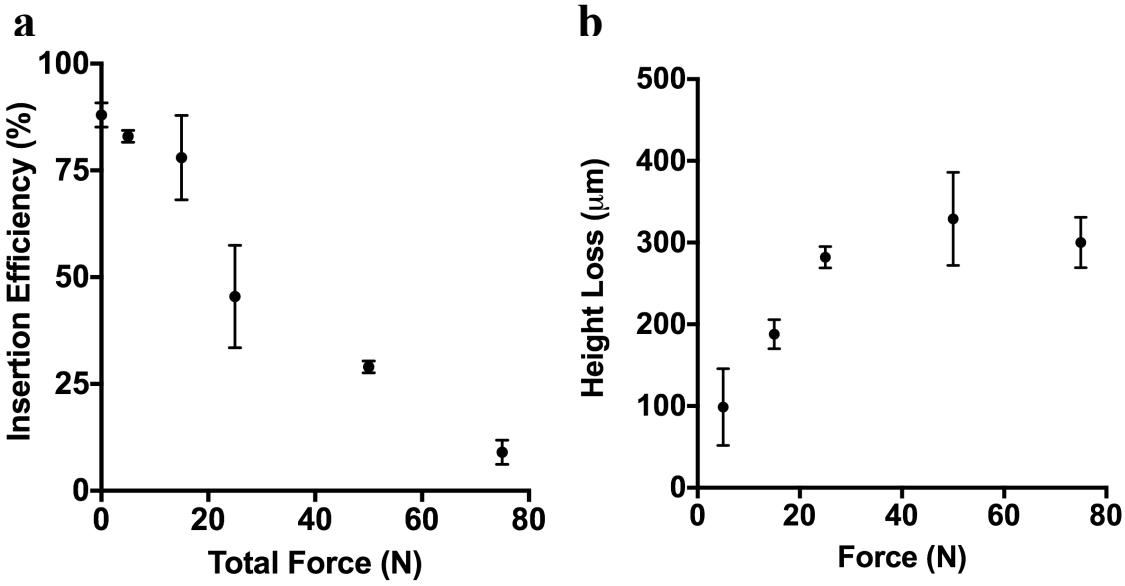

Figure 8: (a) Insertion efficiency for applied forces to a dissolving microneedle. Total applied force was divided by the amount of microneedles in a 10x10 array. (b) Estimated microneedle height loss.

4.1.2 Theoretical Calculations of Microneedle Failure Force

To better understand the observed microneedle mechanics, we sought to model the microneedle behavior. Critical force loads were of primary interest, so we modeled them using the Euler formula for buckling. We felt the Euler formula was most appropriate to model microneedles under compression during application to a rigid surface (i.e., what we did in our experiments) based on a comparison between our needle's column constant and slenderness ratio, as described in Park, et. al[51] (see Appendix A).

Table 1: Predicted critical force values for a 10x10 microneedle patch according to Euler's Formula.

	Critical Force (N)
Fixed-Fixed	2.0
Pinned-Fixed	1.0
Pinned-Pinned	0.5

Using the Euler formula, we evaluated critical force loads for three possible scenarios: fixed-fixed, pinned-pinned and fixed-pinned end (see Appendix A). Estimates from Euler's formula calculation range from 0.5-2.0 N (Table 1). This is in good agreement with the failure force of 1.1 ± 0.6 N observed experimentally. Since there is no rotation occurring at either end of the microneedle, we consider fixed-fixed to be the more appropriate scenario to use for future estimations of microneedle failure force. Ultimately, although microneedles have a varying diameter, the mechanical behavior of our microneedle patches can be studied by using the Euler formula, which treats individual microneedles as columns.

4.2 Immunogenicity in Mice

As a first assessment of the immunogenicity of HBsAg administered using microneedle patches, we evaluated immune response to vaccination with coated microneedle patches as well as dissolvable microneedle patches to administer two doses HBsAg (separated by three weeks) to mice. The study included five groups. Two groups

were vaccinated using coated or dissolvable microneedle patches, and three groups were vaccinated intramuscularly. One of the intramuscular groups administered vaccine as received from the manufacturer. The other two groups received vaccine reconstituted from coated or dissolvable microneedle patches. The reason for including these last two groups was to determine if anything associated with the microneedle patch manufacturing process affected vaccine immunogenicity (independent of the microneedle patch route of administration to the skin).

We characterized the immune response in terms of seroprotection (i.e., anti-HBs > 12 mIU/mL)[63] (Figure 9). Two weeks after administration of the first vaccine dose, 38% of mice in the dissolving microneedle patch group achieved seroprotection, but none of the other groups had any seropositive animals.

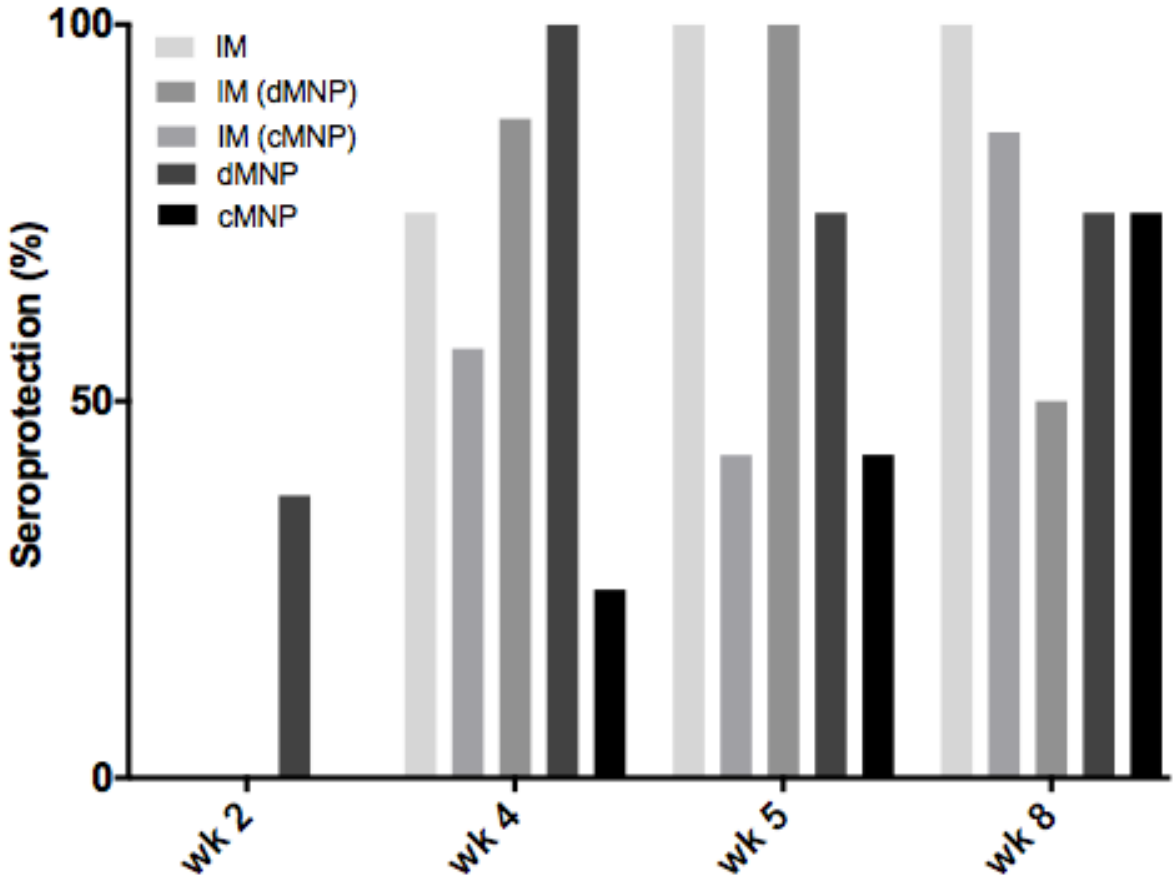

Figure 9: Seroprotection for dissolvable microneedle (dMNP), unadjuvanted bulk antigen (IM) and reconstituted dissolvable microneedle patches (IM (dMNP)) groups. Animals were vaccinated at week 0 and again at week 3.

One week after the second vaccination (i.e., four weeks into the study), all groups had at least 50% seroprotection, except the coated microneedle patch group that had only 25% seroprotected animals. At the end of the study after 8 weeks (i.e., five weeks after the second vaccine dose), all groups had at least 50% seropositive animals (although animals that received the reconstituted dissolvable microneedle patch had only 50% seroprotection). Overall, we can conclude that HBsAg vaccine administered by coated or dissolvable microneedle patches is immunogenic in mice.

Considering safety, no adverse events were noted in any of the animals. At the site of vaccination, the microneedle patches were well tolerated, with no bleeding or erythema observed; in contrast a small drop of blood was sometimes seen at the sites of hypodermic injection.

4.3 Immunogenicity in Macaques

Guided by the immunogenicity results in mice, we vaccinated rhesus macaques with two doses of HBsAg vaccine (separated by nine weeks) as either adjuvanted (commercial formulation provided by Serum Institute of India) or unadjuvanted vaccine via intramuscular injection or unadjuvanted vaccine by dissolvable microneedle patch. Blood draws at weeks -1 and -2 confirmed no initial presence of anti-HBs antibodies (anti-HBs < 1 mIU/mL).

Local reactions to microneedle patch vaccination were mild (Figure 10). Immediately after removing the patch, there was faint evidence of the site where the microneedles had penetrated the skin (square shape in Figure 10b) and where the adhesive had contacted the skin (round shape in Figure 10b) during patch application. No erythema or other signs of irritation was observed. In about 10% of the patches, a speck of blood was seen on the residual patch post-administration. Macaque weights were monitored during the study, and only one macaque showed significant (13%) body weight loss during the course of the study (this animal was in the dMNP group). No other adverse health effects were noted by the veterinary staff.

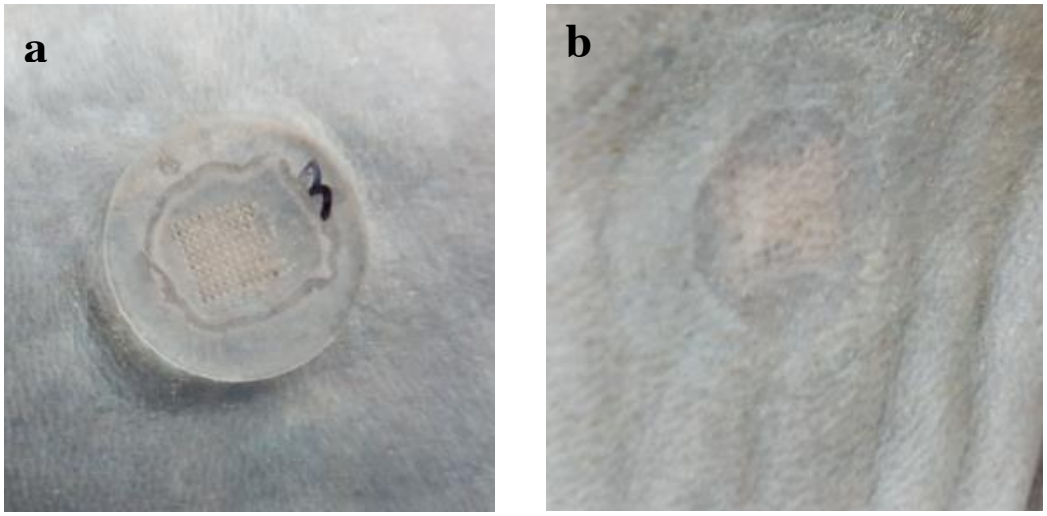

Figure 10: Microneedle patch application on rhesus macaque. Patches were applied to shaved back skin and left on for 20 minutes. Images show a) patch in place b) macaque skin immediately after patch removal.

After administration of the first vaccine dose, all groups showed at least partial seroprotection: 100% in the adjuvanted intramuscular group, 75% in the microneedle patch group and 50% in the nonadjuvanted intramuscular group (Figure 11). After the second dose, macaques vaccinated intramuscularly with the commercial adjuvanted formulation continued to maintain 100% seroprotection until the end of the study. Animals receiving the unadjuvanted vaccine intramuscularly achieved 100% seroprotection the week after the second dose (week 10) and then dropped to 75% seropositive by week 17. The (unadjuvanted) microneedle group achieved 100% seroprotection after the second dose (week 10) and maintained it at week 17.

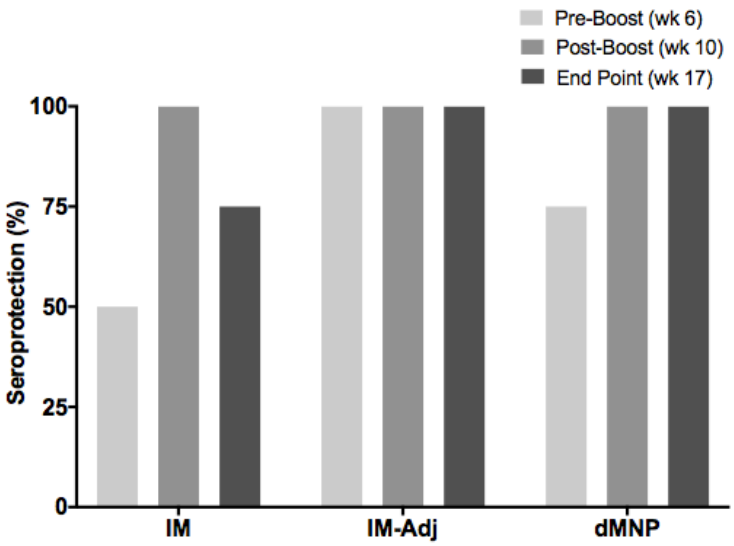

Figure 11: Seroprotection for bulk antigen (IM), adjuvanted antigen formulation (IM-adj) and dissolvable microneedle patch (dMNP) macaque groups.

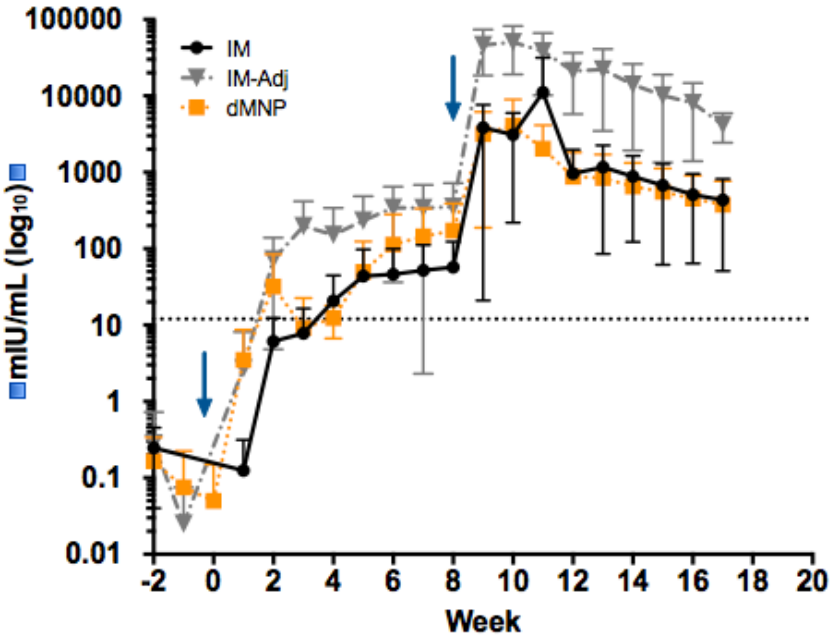

Figure 12: Mean anti-HBc antibody for macaques. Arrows indicate vaccine administration times. Results are shown as mean +/- SD (n=4 for all groups until week 8. dMNP group was n=3 for weeks > 8, due to non-study-related loss of an animal). Dotted line indicates threshold for seroprotection.

The immune response can be further examined by looking at the anti-HBs IgG titers after vaccination (Figure 12). Immediately after the first vaccination (weeks 1 and 2), there was no significant difference in the average antibody titers among the three groups ($P > 0.05$). After that, the titers in all groups roughly maintained their values, and microneedle patch group response by week 8 was not significantly different from the intramuscular groups ($P > 0.05$).

After the second vaccine dose, the microneedle patch and unadjuvanted intramuscular vaccine groups generated almost identical responses with initially increased titers that slowly decayed over time. The adjuvanted intramuscular vaccine led to a significantly greater increase in titers after the second vaccination dose ($P < 0.0001$), but also decayed in value over time. Overall, this study shows that HBsAg vaccination using a microneedle patch is immunogenic in a nonhuman primate model (rhesus macaque).

While we only discussed one microneedle patch group in the above analysis, there were actually two microneedle patch groups in the study: one received a lower dose, which was already presented, and the other received twice the dose, which has not yet been discussed. The high-dose group presented anomalous behavior, with two monkeys that responded to vaccination with only low titers and the other two monkeys were complete non-responders to microneedle patch vaccination, even after two doses. Furthermore, after completion of our study, the non-responsive macaques were vaccinated with the commercial adjuvanted formulation intramuscularly and only then was seroprotection obtained (data not shown). We are not able to distinguish whether the animals had been primed by the microneedle patches or whether this was a primary

response to IM vaccination. These strange data (i.e., much weaker responses compared to the low-dose microneedle patch and both intramuscular groups) may be related to patch function or variability within the small sample size of outbred animals.

For that reason, we present the data including this group in Appendix B, but refrain from discussing it in the main body of the thesis.

CHAPTER 5. DISCUSSION

5.1 Mechanical Characterization of Microneedles

Effective microneedle insertion into skin relies on parameters like needle geometry, needle aspect ratio and array size. Microneedle patch design must take into account these factors as well as others like microneedle density, as this can lead to issues like the "bed of nails" effect where a tightly packed array acts as a single entity causing improper skin deflection. In the case of dissolvable microneedles, it is even more crucial to ensure array design facilitates insertion. By using an entire microneedle array during force testing - as opposed to a single microneedle - we hope to capture the behavior most relevant for the insertion process for needles of this geometry.

Previous works have explored the effect of different base geometries[64], as well as aspect ratio of microneedles.[51] Previous work has determined the failure force of 3 x 3 conical microneedle arrays made out of CMC.[24] Here, we sought to characterize the mechanical properties of a 10 x 10 dissolvable microneedle patch with conical microneedles having a height of 600 μm. In addition, the loss of microneedle volume due to crushing was characterized in order to demonstrate the force that can be applied without a significant loss of insertion efficiency.

A brittle failure point was never observed under compression for the 10 x 10 dMNPs under the conditions studied. It is certainly possible that under non-axial force loading situations the microneedle patch could experience sudden brittle failure or even a plateauing indicating ductile failure. However, it ultimately is clear from the force-

displacement curves as well as from imaged patches that the microneedles in this study fail through yielding. It is well documented that residual water content in polymers acts as a plasticizer, and therefore it is likely that the observed behavior is a function of residual moisture in the microneedle patch.

The data obtained through force-displacement curves suggest that the current microneedle patch design starts to fail at 11 ± 6 mN per microneedle. It has been shown by others that conical polyvinylpyrrolidone (PVP) microneedles and CMC microneedles (15 x 15 and 3 x 3 arrays, respectively) had failure forces at ~100 mN/needle.[29,65] Given the different materials and microneedle patch design, we do only expect rough agreement with these studies. Furthermore, a study by Park et. al found that conical poly(lactic-co-glycolic acid) (PLGA) microneedles failed at around 175 μm of compression for MNs with a height of 570 μm. These findings are comparable to our observation that our microneedles with a height of 600 μm failed at 170 ± 70 μm of compression. Despite these reported failure forces, it is of interest to note that insertion into skin was still possible in this study at forces three times higher than the observed force for failure, meaning that this failure force is not the maximum force that a microneedle can tolerate before being made useless for insertion into skin, but that larger forces can be tolerated while still permitting microneedle penetration into skin.

Microneedle patches were kept in a sealed, dark pouch with desiccant until use in this study. The failure force exhibited by the dMNPs varied by 50% (i.e., 11 ± 6 mN per microneedle at yield point), and this may have been affected by the length of time the microneedle patches were kept in storage before use. It is possible that variability in

failure force was due to insufficiently controlled microneedle water content due to humidity changes over time after removal from the desiccated storage pouch.

5.2 Immune Response to HBsAg Vaccination using Microneedle Patches

The main goal of this study was to assess the immunogenicity of dissolving microneedle patches containing HBV vaccine in both mice and rhesus macaques. We showed via in vivo studies that skin vaccination using our current formulation for dissolvable microneedle patches elicited a fast and sustained immune response that was comparable to the current intramuscular vaccine. Notably, in our mouse study, protective immune response started developing two weeks faster than via intramuscular administration.

Interestingly, in our macaque study half of our high-dose group was non-responsive, and the other half had titers that although seroprotective, were much lower than those of the low-dose microneedle group. It remains unclear why this occurred, as patches were shown to be antigenic in vitro. Nonetheless, 6 out of 8 macaques that received patch vaccine generated anti-HBs antibodies. Overall, this work shows that microneedle patches can successfully deliver hepatitis B antigen to generate a strong and sustained immune response in more than one animal model.

5.3 Potential Impact on Public Health

Vaccination against HBV is the principal way to address and control the long-term health and economical burdens associated with this disease. Although in 1991 the World Health Organization recommended a universal hepatitis B vaccination policy to be effective by

1997, its implementation was hampered for many years by the high cost of HBV vaccine compared to other Expanded Program on Immunizations (EPI) vaccines.[8] With funding from sources like GAVI, the addition of HBV vaccine as part of the routine infant immunization schedule and dramatically cheaper vaccine offered by developing country vaccine manufacturers have boosted coverage significantly, efforts are needed to address populations not currently benefitting from current public health policy[7.] Currently, the countries with least HBV vaccination coverage suffer from disadvantaged economies [8] and limited health-care infrastructure. The possibility of an inexpensive, easy to distribute vaccine holds great potential to overcome the financial and logistical hurdles associated with vaccination campaigns.

The current design for dissolvable microneedle patches has been shown to allow for administration without the use of an applicator or advanced training.[22] Because of the CDC recommendation that first administration occur at birth, this mode of vaccination could be of particular benefit in cases where cultural preference or lack of resources result in a non-hospital birth. A microneedle patch would facilitate vaccination in these cases without the need for additional medical intervention. Finally, microneedle patches are lightweight, and could cut down on the wastage produced with the current multi-dose vials. Further development of this technology would open up the possibility to distribute and administer in remote locations, and optimize the efforts of health workers spread thin by current vaccination campaigns.

CONCLUSION

In this work, we developed dissolvable microneedle patches that administer HBV vaccine (HBsAg) and evaluated their immunogenicity in BALB/c mice as well as rhesus macaques. These vaccine patches were found to be immunogenic, and 75% seroprotection was achieved for the rhesus macaque model. Moreover, the data shown in this work suggests that dissolving microneedle patches fail by yielding as opposed to brittle fracture, but also that limited minor microneedle tip deformation may not have a significant effect on insertion. Ultimately, microneedle patches present many logistical advantages for improving vaccination coverage worldwide, by offering an easy alternative for infant vaccination that can be used in births that occur in remote, non-hospital settings.

RECOMMENDATIONS

These studies confirmed that HBV vaccine could be encapsulated within a dissolving microneedle patch to elicit a robust immune response in two different pre-clinical animal models. Future work should center on improving vaccine stability, with consideration to different temperature and humidity conditions. This can be achieved by performing an expansive screening of excipients. It is of importance to find long-term thermostability because it would be a substantial benefit to make this kind of technology more economical to implement at a large scale.

Another focus of future work should be the evaluation of the possibility of dose sparing even without adjuvantation. Additional research can assess this by additional *in vivo* studies that further optimize vaccine stability and delivery from the microneedle patch and incorporate dosing regimens lower than the typical (10 μg - 20 μg) full human dose.

APPENDIX A. THEORETICAL CALCULATIONS FOR CRITICAL FORCE

The slenderness ratio of a column is calculated by:

$$SR = \frac{KL}{D/4}$$

SR values for our microneedles were 26.7, 18.7 and 13.3 using K values of 1.0, 0.7 and 0.5, respectively (see sample calculation below).

$$SR = \frac{KL}{D/4} = \frac{1.0\ (600)\mu m}{90\ \mu m/4} = \ 26.\overline{6}$$

To confirm that Euler's formula applied to our geometry, the column constant was calculated and compared to our slenderness ratio values. The column constant provides a transition point to determine whether the column is considered "short" or "long" (and therefore whether Euler's formula applies)

$$Column\ constant = \sqrt{\frac{2\pi^2 E}{S_y}} = 4.45$$

For all K values studied, the slenderness ratio is larger than the column constant, and so Euler's formula is appropriate to use.

Table A1: Parameters used to calculate critical load via Euler's equation

Parameter		Units
Yield Strength (S_y)	5.66×10^9 [19]	Pa
Equivalent Diameter (D_{eq})	90	µm
E	5.69×10^9	Pa
K	0.5,0.7 or 1.0	n/a
microneedle height (L)	6.00×10^{-6}	m
critical load (F_{cr})		N

To determine the critical load for each scenario, the corresponding effective length (L_e) is obtained by multiplying microneedle height (L) and K. Critical load is determined by plugging in the values from Table A1 into the following equation:

$$\boldsymbol{F_{cr} = \frac{\pi^2 EI}{L_e^2}}$$

Where the moment of inertia is calculated using the following equation:

$$I = \frac{\pi {D_{eq}}^4}{64}$$

APPENDIX B. MACAQUE ANTIBODY DATA

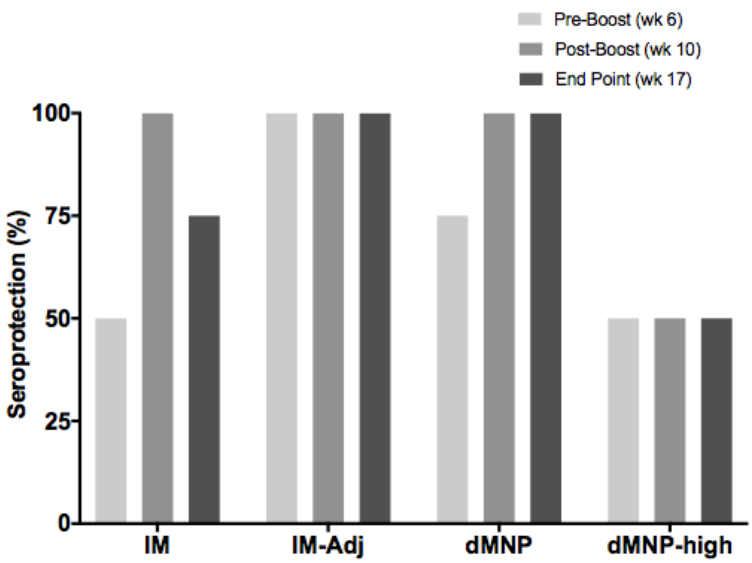

Figure B1: Seroprotection for bulk antigen (IM) and adjuvanted antigen formulation (IM-adj) and low and high dose dissolving microneedles (dMNP and dMNP-high) macaque groups.

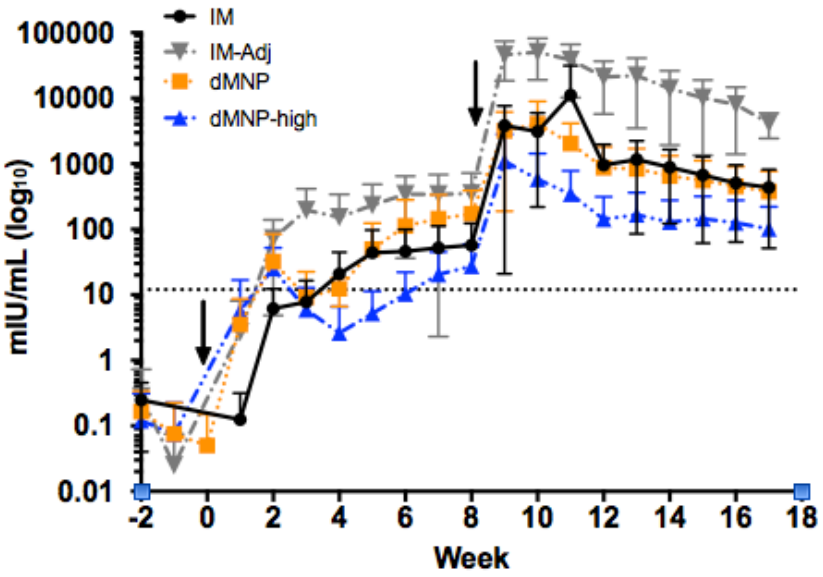

Figure B2: Mean anti-HBc antibody for macaques. Arrows indicate vaccine administration. Data includes low and non-responders in the MN-high group. Results are shown as mean +/- SD (n=4 for all groups until week 8. dMNP group was n=3 for weeks > 8). Dotted line indicates threshold for seroprotection.

REFERENCES

(1) Lavanchy, D.; Kane, M. Global Epidemiology of Hepatitis B Virus Infection. In *Hepatitis B Virus in Human Diseases*; 2016; pp. 187–203.

(2) Immunization Coverage- Hepatitis B http://www.who.int/mediacentre/factsheets/fs378/en/.

(3) Damme, P. Van; Ward, J.; Shouval, D.; Wiersma, S. Hepatitis B Vaccines. In; 2013; pp. 205–234.

(4) Arya, J.; Prausnitz, M. R. Microneedle Patches for Vaccination in Developing Countries. *J. Control. Release* **2015**, *240*, 135–141.

(5) Montagu, D.; Yamey, G.; Visconti, A.; Harding, A.; Yoong, J. Where Do Poor Women in Developing Countries Give Birth? A Multi-Country Analysis of Demographic and Health Survey Data. *PLoS One* **2011**, *6*.

(6) Lala, M. K.; Lala, K. R. Thermostability of Vaccines. *Indian Pediatr.* **2003**, *40*, 311–319.

(7) Chen, D.; Kapre, S.; Goel, a.; Suresh, K.; Beri, S.; Hickling, J.; Jensen, J.; Lal, M.; Preaud, J. M.; LaForce, M.; *et al.* Thermostable Formulations of a Hepatitis B Vaccine and a Meningitis A Polysaccharide Conjugate Vaccine Produced by a Spray Drying Method. *Vaccine* **2010**, *28*, 5093–5099.

(8) Zanetti, A. R.; Van Damme, P.; Shouval, D. The Global Impact of Vaccination against Hepatitis B: A Historical Overview. *Vaccine* **2008**, *26*, 6266–6273.

(9) Lebre, F.; Borchard, G.; De Lima, M. C. P.; Borges, O. Progress towards a Needle-Free Hepatitis B Vaccine. *Pharm. Res.* **2011**, *28*, 986–1012.

(10) Prevention, C. for D. C. and. Surveillance for Viral Hepatitis - United States, 2013 http://www.cdc.gov/hepatitis/statistics/2013surveillance/commentary.htm#hepatitisC.

(11) Williams, W. W.; Lu, P.-J.; O'Halloran, A.; Kim, D. K.; Grohskopf, L. A.; Pilishvili, T.; Skoff, T. H.; Nelson, N. P.; Harpaz, R.; Markowitz, L. E.; *et al.* Surveillance of Vaccination Coverage Among Adult Populations - United States, 2014. *Mmwr* **2016**, *65*, 1–36.

(12) Hepatitis B Fact Sheet http://www.who.int/mediacentre/factsheets/fs204/en/# (accessed Jun 15, 2015).

(13) *WHO Technical Report Series: Recommendations to Assure the Quality , Safety and Efficacy of Recombinant Hepatitis B Vaccines*; 2013.

(14) Plotkin, S. *Vaccines: Expert Consult - Online and Print*; Plotkin, S.; Orenstein, W.; Offit, P., Eds.; 6th ed.; Elsevier Health Sciences, 2012.

(15) Greiner, V. J.; Manin, C.; Larquet, E.; Ikhelef, N.; Gréco, F.; Naville, S.; Milhiet, P. E.; Ronzon, F.; Klymchenko, A.; Mély, Y. Characterization of the Structural Modifications Accompanying the Loss of HBsAg Particle Immunogenicity. *Vaccine* **2014**, *32*, 1049–1054.

(16) Gómez-Gutiérrez, J.; Rodríguez-Crespo, I.; González-Ros, J. M.; Ferragut, J. a; Paul, D. a; Peterson, D. L.; Gavilanes, F. Thermal Stability of Hepatitis B Surface Antigen S Proteins. *Biochim. Biophys. Acta* **1992**, *1119*, 225–231.

(17) Kumru, O. S.; Joshi, S. B.; Smith, D. E.; Middaugh, C. R.; Prusik, T.; Volkin, D. B. Vaccine Instability in the Cold Chain: Mechanisms, Analysis and Formulation Strategies. *Biologicals* **2014**, *42*, 237–259.

(18) Organization, W. H. *A SYSTEMATIC REVIEW OF MONOVALENT HEPATITIS B VACCINE*; 2016.

(19) Tonnis, W. F.; Amorij, J. P.; Vreeman, M. a.; Frijlink, H. W.; Kersten, G. F.; Hinrichs, W. L. J. Improved Storage Stability and Immunogenicity of Hepatitis B Vaccine after Spray-Freeze Drying in Presence of Sugars. *Eur. J. Pharm. Sci.* **2014**, *55*, 36–45.

(20) Loizidou, E. Z.; Williams, N. A.; Barrow, D. A.; Eaton, M. J.; McCrory, J.; Evans, S. L.; Allender, C. J. Structural Characterisation and Transdermal Delivery Studies on Sugar Microneedles: Experimental and Finite Element Modelling Analyses. *Eur. J. Pharm. Biopharm.* **2015**, *89*, 224–231.

(21) Kim, Y. C.; Park, J. H.; Prausnitz, M. R. Microneedles for Drug and Vaccine Delivery. *Adv. Drug Deliv. Rev.* **2012**, *64*, 1547–1568.

(22) Norman, J. J.; Arya, J. M.; McClain, M. A.; Frew, P. M.; Meltzer, M. I.; Prausnitz, M. R. Microneedle Patches: Usability and Acceptability for Self-Vaccination against Influenza. *Vaccine* **2014**, *32*, 1856–1862.

(23) Kim, Y.-C.; Park, J.-H.; Prausnitz, M. R. Microneedles for Drug and Vaccine Delivery. **2013**, *64*, 1547–1568.

(24) Prausnitz, M. R. Microneedles for Transdermal Drug Delivery. *Adv. Drug Deliv. Rev.* **2004**, *56*, 581–587.

(25) Lee, J. W.; Choi, S. O.; Felner, E. I.; Prausnitz, M. R. Dissolving Microneedle Patch for Transdermal Delivery of Human Growth Hormone. *Small* **2011**, *7*, 531–539.

(26) Engelke, L.; Winter, G.; Hook, S.; Engert, J. Recent Insights into Cutaneous Immunization: How to Vaccinate via the Skin. *Vaccine* **2015**, *33*, 4663–4674.

(27) Prausnitz, M. R.; Mikszta, J. A.; Cormier, M.; Andrianov, A. K. Microneedle-Based Vaccines. In *Vaccines for Pandemic Influenza*; 2009; Vol. 333, pp. 369–393.

(28) Prausnitz, M. R.; Langer, R. Transdermal Drug Delivery. *Nat. Biotechnol.* **2009**, *26*, 1261–1268.

(29) Lee, J. W. Physical Enhancement of Transdermal Drug Delivery : Polysaccharide Dissolving Microneedles and Micro Thermal Skin Ablation Physical Enhancement of Transdermal Drug Delivery : Polysaccharide Dissolving Microneedles and, 2009.

(30) Larrañeta, E.; Lutton, R. E. M.; Woolfson, A. D.; Donnelly, R. F. Microneedle Arrays as Transdermal and Intradermal Drug Delivery Systems: Materi(1) Larrañeta, E.; Lutton, R. E. M.; Woolfson, A. D.; Donnelly, R. F. Microneedle Arrays as Transdermal and Intradermal Drug Delivery Systems: Materials Science, Manufacture an. *Mater. Sci. Eng. R Reports* **2016**, *104*, 1–32.

(31) Hirobe, S.; Azukizawa, H.; Hanafusa, T.; Matsuo, K.; Quan, Y. S.; Kamiyama, F.; Katayama, I.; Okada, N.; Nakagawa, S. Clinical Study and Stability Assessment of a Novel Transcutaneous Influenza Vaccination Using a Dissolving Microneedle Patch. *Biomaterials* **2015**, *57*, 50–58.

(32) Van Der Maaden, K.; Trietsch, S. J.; Kraan, H.; Varypataki, E. M.; Romeijn, S.; Zwier, R.; Van Der Linden, H. J.; Kersten, G.; Hankemeier, T.; Jiskoot, W.; *et al.* Novel Hollow Microneedle Technology for Depth-Controlled Microinjection-Mediated Dermal Vaccination: A Study with Polio Vaccine in Rats. *Pharm. Res.* **2014**, *31*, 1846–1854.

(33) Cary, D. *FDA Licenses Sanofi Pasteur ' S New Influenza Vaccine Delivered by Intradermal Microinjection an Immunization Option with 90 Percent Smaller Needle*; 2011.

(34) Edens, C.; Collins, M. L.; Goodson, J. L.; Rota, P. A.; Prausnitz, M. R. A Microneedle Patch Containing Measles Vaccine Is Immunogenic in Non-Human Primates. *Vaccine* **2015**, *33*, 4712–4718.

(35) Mistilis, M. J.; Bommarius, A. S.; Prausnitz, M. R. Development of a Thermostable Microneedle Patch for Influenza Vaccination. *J. Pharm. Sci.* **2015**, *104*, 740–749.

(36) Arya, J. M.; Dewitt, K.; Scott-Garrard, M.; Chiang, Y. W.; Prausnitz, M. R. Rabies Vaccination in Dogs Using a Dissolving Microneedle Patch. *J. Control. Release* **2016**, *239*, 19–26.

(37) Mistilis, M. J.; Joyce, J. C.; Esser, E. S.; Skountzou, I.; Compans, R. W.; Bommarius, A. S.; Prausnitz, M. R. Long-Term Stability of Influenza Vaccine in a Dissolving Microneedle Patch. *Drug Deliv. Transl. Res.* **2016**, 1–11.

(38) Center for Disease Control and Prevention. A Comprehensive Immunization Strategy to Eliminate Transmission of Hepatitis B Virus Infection in the United States Recommendations of the Advisory Committee on Immunization Practices (ACIP) Part 1: Immunization of Infants, Children, and Adolescents. *MMWR Recomm. Reports* **2005**, *54*, 1–32.

(39) Hui, D. Hepatitis B. *Approach to Intern. Med. a Resour. B. Clin. Pract.* **2011**, 130–131.

(40) Moon, S.; Wang, Y.; Edens, C.; Gentsch, J. R.; Prausnitz, M. R.; Jiang, B. Dose Sparing and Enhanced Immunogenicity of Inactivated Rotavirus Vaccine Administered by Skin Vaccination Using a Microneedle Patch. *Vaccine* **2013**, *31*, 3396–3402.

(41) Van Damme, P.; Oosterhuis-Kafeja, F.; Van der Wielen, M.; Almagor, Y.; Sharon, O.; Levin, Y. Safety and Efficacy of a Novel Microneedle Device for Dose Sparing Intradermal Influenza Vaccination in Healthy Adults. *Vaccine* **2009**, *27*, 454–459.

(42) La Montagne, J. R.; Fauci, A. S. Intradermal Influenza Vaccination--Can Less Be More? *N. Engl. J. Med.* **2004**, *351*, 2330–2332.

(43) Goncalves, L.; Albarran, B.; Salmen, S.; Borges, L.; Fields, H.; Montes, H.; Soyano, A.; Diaz, Y.; Berrueta, L. The Nonresponse to Hepatitis B Vaccination Is Associated with Impaired Lymphocyte Activation. *Virology* **2004**, *326*, 20–28.

(44) Tajiri, K.; Shimizu, Y. Unsolved Problems and Future Perspectives of Hepatitis B Virus Vaccination. *World J. Gastroenterol.* **2015**, *21*, 7074–7083.

(45) Ploemen, I. H. J.; Hirschberg, H. J. H. B.; Kraan, H.; Zeltner, A.; Van Kuijk, S.; Lankveld, D. P. K.; Royals, M.; Kersten, G. F. A.; Amorij, J. P. Minipigs as an Animal Model for Dermal Vaccine Delivery. *Comp. Med.* **2014**, *64*, 50–54.

(46) Hirschberg, H.; Van Kuijk, S.; Loch, J.; Jiskoot, W.; Bouwstra, J.; Kersten, G.; Amorij, J. P. A Combined Approach of Vesicle Formulations and Microneedle Arrays for Transcutaneous Immunization against Hepatitis B Virus. *Eur. J. Pharm. Sci.* **2012**, *46*, 1–7.

(47) Guo, L.; Qiu, Y.; Chen, J.; Zhang, S.; Xu, B.; Gao, Y. Effective Transcutaneous Immunization against Hepatitis B Virus by a Combined Approach of Hydrogel Patch Formulation and Microneedle Arrays. *Biomed. Microdevices* **2013**, *15*, 1077–1085.

(48) Mikszta, J. a; Alarcon, J. B.; Brittingham, J. M.; Sutter, D. E.; Pettis, R. J.; Harvey, N. G. 28-Improved Genetic Immunization via Micromechanical Disruption of Skin-Barrier Function and Targeted Epidermal Delivery. *Nat. Med.* **2002**, *8*, 415–419.

(49) Andrianov, A. K.; DeCollibus, D. P.; Gillis, H. a; Kha, H. H.; Marin, A.; Prausnitz, M. R.; Babiuk, L. a; Townsend, H.; Mutwiri, G. Poly[di(carboxylatophenoxy)phosphazene] Is a Potent Adjuvant for Intradermal Immunization. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106*, 18936–18941.

(50) Qiu, Y.; Guo, L.; Mao, P.; Gao, Y. Dissolving Microneedle Arrays for Intradermal Immunization of Hepatitis B Virus DNA Vaccine. *Procedia Vaccinol.* **2015**, *9*, 24–30.

(51) Jung-Hwan, P.; Mark, P. Analysis of Mechanical Failure of Polymer Microneedles by Axial Force. *J. Korean Phys. Soc.* **2010**, *56*, 1223.

(52) Pilkey, W. D. *Formulas for Stress, Staind, and Structural Matrices*; 1994.

(53) Roark, R. J.; Young, W. C.; Plunkett, R. *Formulas for Stress and Strain*; 1976; Vol. 43.

(54) van der Maaden, K.; Sekerdag, E.; Jiskoot, W.; Bouwstra, J. Impact-Insertion Applicator Improves Reliability of Skin Penetration by Solid Microneedle Arrays. *AAPS J.* **2014**, *16*, 681–684.

(55) Davis, S. P.; Landis, B. J.; Adams, Z. H.; Allen, M. G.; Prausnitz, M. R. Insertion of Microneedles into Skin: Measurement and Prediction of Insertion Force and Needle Fracture Force. *J. Biomech.* **2004**, *37*, 1155–1163.

(56) Lee, J. W.; Park, J. H.; Prausnitz, M. R. Dissolving Microneedles for Transdermal Drug Delivery. *Biomaterials* **2008**, *29*, 2113–2124.

(57) Sullivan, S. P.; Koutsonanos, D. G.; Del Pilar Martin, M.; Lee, J. W.; Zarnitsyn, V.; Choi, S.-O.; Murthy, N.; Compans, R. W.; Skountzou, I.; Prausnitz, M. R. Dissolving Polymer Microneedle Patches for Influenza Vaccination. *Nat. Med.* **2010**, *16*, 915–920.

(58) Spradling, P. R.; Xing, J.; Williams, R.; Masunu-Faleafaga, Y.; Dulski, T.; Mahamud, A.; Drobeniuc, J.; Teshale, E. H. Immunity to Hepatitis B Virus (HBV) Infection Two Decades after Implementation of Universal Infant HBV Vaccination: Association of Detectable Residual Antibodies and Response to a Single HBV Challenge Dose. *Clin. Vaccine Immunol.* **2013**, *20*, 559–561.

(59) Development, A.; Hepatitis, V.; Kamili, S. Laboratory Procedure Manual Hepatitis B Surface Antibody (Anti-HBS) Serum. **2013**, 1–18.

(60) Vassilieva, E. V.; Kalluri, H.; McAllister, D.; Taherbhai, M. T.; Esser, E. S.; Pewin, W. P.; Pulit-Penaloza, J. A.; Prausnitz, M. R.; Compans, R. W.; Skountzou, I. Improved Immunogenicity of Individual Influenza Vaccine Components Delivered with a Novel Dissolving Microneedle Patch Stable at Room Temperature. *Drug Deliv. Transl. Res.* **2015**, *5*, 360–371.

(61) Edens, C.; Collins, M. L.; Ayers, J.; Rota, P. A.; Prausnitz, M. R. Measles Vaccination Using a Microneedle Patch. *Vaccine* **2013**, *31*, 3403–3409.

(62) Gill, H. S. Coated Microneedles and Microdermabrasion for Transdermal Delivery. *Microbiol. Immunol.* **2007**.

(63) User, C. D. C. Laboratory Procedure Manual Hepatitis B Surface Antigen (HBsAg) Serum. **2013**.

(64) Loizidou, E. Z.; Inoue, N. T.; Ashton-Barnett, J.; Barrow, D. A.; Allender, C. J. Evaluation of Geometrical Effects of Microneedles on Skin Penetration by CT Scan and Finite Element Analysis. *Eur. J. Pharm. Biopharm.* **2016**, *107*, 1–6.

(65) Sullivan, S. P.; Murthy, N.; Prausnitz, M. R. Minimally Invasive Protein Delivery with Rapidly Dissolving Polymer Microneedles. *Adv. Mater.* **2008**, *20*, 933–938.